

METHODS AND COMPOSITIONS FOR MODULATING AXONAL OUTGROWTH OF CENTRAL NERVOUS SYSTEM NEURONS

Background of the Invention

Past early childhood, injury to the central nervous system (CNS) results in functional impairments that are largely irreversible. Within the brain or spinal cord, damage resulting from stroke, trauma, or other causes can result in life-long losses in cognitive, sensory and motor functions, and even maintenance of vital functions. Nerve cells that are lost are not replaced, and those that are spared are generally unable to re-grow severed connections, although a limited amount of local synaptic reorganization can occur close to the site of injury. Functions that are lost are currently untreatable.

Regenerative failure in the CNS has been attributed to a number of factors, which include the presence of inhibitory molecules on the surface of glial cells that suppress axonal growth; absence of appropriate substrate molecules such as laminin to foster growth and an absence of the appropriate trophic factors needed to activate programs of gene expression required for cell survival and differentiation.

By contrast, within the peripheral nervous system (PNS), injured nerve fibers can re-grow over long distances, with eventual excellent recovery of function. Within the past 15 years, neuroscientists have come to realize that this is not a consequence of intrinsic differences between the nerve cells of the peripheral and central nervous system; remarkably, neurons of the CNS will extend their axons over great distances if given the opportunity to grow through a grafted segment of PNS (*e.g.*, sciatic nerve). Therefore, neurons of the CNS retain a capacity to grow if given the right signals from the extracellular environment. Factors which contribute to the differing growth potentials of the CNS and PNS include certain growth-inhibiting molecules on the surface of the oligodendrocytes that surround nerve fibers in the CNS, but which are less abundant in the comparable cell population of the PNS (Schwann cells); molecules of the basal lamina and other surfaces that foster growth in the PNS but which are absent in the CNS (*e.g.*, laminin); and trophic factors, soluble polypeptides which activate programs of gene expression that underlie cell survival and differentiation. Although such trophic factors are regarded as essential for maintaining the viability and differentiation of nerve cells, the particular ones that are responsible for inducing axonal regeneration in the CNS remain uncertain.

Moreover, the intracellular molecule(s) that mediates axonal outgrowth of normal neuronal cells (*e.g.*, upon stimulation with extracellular factors or upstream

secondary messengers) has not been elucidated. One report has described the partial isolation of a kinase, referred to as "protein kinase N", from rat pheochromocytoma PC12 cells that is activated by NGF treatment of the PC12 cells and sensitive to purine regulation (C. Volonte, *et al.*, (1989) *J. Cell Biol.* 109, 2395-403). However, as PC12
5 cells are a rat pheochromocytoma cell line from the adrenal medulla, with many different properties than normal CNS neurons, these cells present a limited model for the processes by which growth of normal CNS neurons is stimulated and the results obtained in PC12 cells may not be predictive of molecules involved in normal CNS neuron growth. Furthermore, this protein kinase N was only partially purified and
10 remains to be molecularly characterized. .

In view of the lack of understanding of the molecules involved in mediating axonal outgrowth, effective treatments for CNS injuries have not been developed. Accordingly, elucidation and molecular characterization of such molecules is still necessary, and methods and compositions for modulating the outgrowth of normal CNS
15 neurons by modulating the activity of such molecules are still needed.

Summary of the Invention

The present invention provides methods and compositions for modulating the axonal outgrowth of central nervous system neurons, in particular normal mammalian
20 central nervous system neurons. The invention is based, at least in part, on the isolation of a highly purified form of the N-kinase polypeptide from normal mammalian neuronal tissue, molecular characterization of its chemical structure (including amino acid sequence), demonstration of its sensitivity to purine regulation and the discovery that this kinase plays an active role in the axonal outgrowth of CNS neurons, including
25 mammalian CNS neurons, such as retinal ganglion neurons. Identification of N-kinase as a critical intracellular mediator of axonal outgrowth, and chemical characterization of its structure, now provides for the ability to modulate axonal outgrowth by modulating N-kinase activity. Furthermore, this purification and characterization of N-kinase now allows for its use in screening assays to identify additional modulators of axonal
30 outgrowth.

Accordingly, in one aspect, the present invention is directed to a method for treating a subject (*e.g.*, a mammal, such as a human) suffering or prone to suffering from a condition characterized by aberrant, *e.g.*, inadequate or insufficient, axonal outgrowth of central nervous system neurons (*e.g.*, stroke, CNS trauma, or a neurodegenerate
35 disease), by administering to the subject a compound that modulates the activity of N-kinase, thereby treating the subject suffering or prone to suffering from a condition characterized by aberrant axonal outgrowth of central nervous system neurons.

The methods of the invention for modulating, *e.g.*, stimulating, the axonal outgrowth of central nervous system neurons can be used following damage or other injury to the CNS neurons (*e.g.*, stroke, Traumatic Brain Injury, cerebral aneurism, spinal cord injury and the like). The methods of the invention for modulating, *e.g.*, inhibiting, the axonal outgrowth of CNS neurons can be used in neuroproliferative disorders where aberrant axonal outgrowth may occur, such as epilepsy (*e.g.*, post-traumatic epilepsy) and neuropathic pain syndrome.

In one aspect, the compound that modulates the activity of N-kinase is administered to a subject in accordance with the present invention by introduction into the central nervous system of the subject, for example into the cerebrospinal fluid of the subject. In certain aspects of the invention, the compound that modulates the activity of N-kinase is introduced intrathecally, for example into a cerebral ventricle, the lumbar area, or the cisterna magna. In a preferred embodiment, the method of the invention modulates outgrowth of damaged cortical neurons. In yet another preferred embodiment, the method of the invention modulates outgrowth of damaged retinal ganglion cells. In such circumstances, the compound that modulates the activity of N-kinase can be administered locally to cortical neurons or retinal ganglion cells to modulate axonal outgrowth.

In yet another aspect of the invention, the compound that modulates the activity of N-kinase is administered in a pharmaceutically acceptable formulation. The pharmaceutically acceptable formulation can be a dispersion system, for example a lipid-based formulation, a liposome formulation, or a multivesicular liposome formulation. The pharmaceutically acceptable formulation can also comprise a polymeric matrix, selected, for example, from synthetic polymers such as polyesters (PLA, PLGA), polyethylene glycol, poloxomers, polyanhydrides, and pluronics or selected from naturally derived polymers, such as albumin, alginate, cellulose derivatives, collagen, fibrin, gelatin, and polysaccharides.

In a preferred embodiment, the pharmaceutically acceptable formulation provides sustained delivery, *e.g.*, "slow release" of the compound that modulates the activity of N-kinase to a subject for at least one week, more preferably at least one month, after the pharmaceutically acceptable formulation is administered to the subject. Preferred approaches for achieving sustained delivery of a formulation of the invention include the use of a slow release polymeric capsules or an infusion pump that includes the formulation.

In one embodiment of the invention, the compound that modulates the activity of N-kinase is a small molecule, the N-kinase polypeptide or fragment thereof, an anti-N-kinase antibody, an antisense N-kinase nucleic acid molecule, a ribozyme, or the N-kinase gene or fragment thereof.

In another aspect, the invention features a method for modulating, *e.g.*, stimulating or inhibiting, axonal outgrowth of a central nervous system neuron (such as a mammalian central nervous system neuron) by contacting the central nervous system neuron with a compound that modulates the activity of N-kinase, thereby modulating axonal outgrowth of the central nervous system neuron.

In yet another aspect, the invention features a method for modulating the axonal outgrowth of a central nervous system neuron in a subject, by administering to the subject a compound that modulates the activity of N-kinase, such that axonal outgrowth in the subject is modulated.

In a further aspect, the invention features a method for identifying a compound that modulates axonal outgrowth of a central nervous system neuron by contacting N-kinase with a test compound and determining the ability of the test compound to modulate the activity of N-kinase, thereby identifying a compound that modulates axonal outgrowth of a central nervous system neuron. In a preferred embodiment, the ability of the test compound to modulate the activity of N-kinase is determined by assessing the ability of the test compound to modulate N-kinase dependent phosphorylation of a substrate, *e.g.*, a histone HF-1 protein.

In one embodiment, the N-kinase used in the methods of the invention is a human N-kinase, such as a recombinantly produced human N-kinase. In another embodiment, the N-kinase used in the methods of the invention is a bovine N-kinase, such as an N-kinase which is purified from a bovine source, *e.g.*, neonatal bovine brain tissue.

In another embodiment, the screening method of the invention further includes determining the ability of the test compound to modulate axonal outgrowth of a central nervous system neuron.

In another aspect, the invention features a method for identifying a compound that modulates axonal outgrowth of a central nervous system neuron, comprising contacting N-kinase with a test compound, an N-kinase substrate (*e.g.*, a histone HF-1 protein), radioactive ATP (*e.g.*, [γ - ^{32}P] ATP), and Mn^{+2} ; and determining the ability of the test compound to modulate N-kinase dependent phosphorylation of the substrate, thereby identifying a compound that modulates axonal outgrowth of a central nervous system neuron. In a preferred embodiment, the method of the invention further includes determining the ability of the test compound to modulate axonal outgrowth of a central nervous system neuron.

In another aspect, the invention features a compound that modulates axonal outgrowth of a central nervous system neuron identified by any of the foregoing methods.

In yet another aspect, the invention features an isolated N-kinase polypeptide of the type that: (a) is present in neonatal brain tissue (*e.g.*, neonatal human, bovine, rat, or mouse brain tissue); (b) is inhibited by 6-thioguanine; (c) is activated by Mn^{+2} but not by Mg^{+2} or Ca^{+2} ; (d) has a molecular weight of approximately 49 kDa; and (e) is eluted from a Cibacron Blue column at a NaCl concentration of 1.5-1.75 M.

In a further aspect, the invention features an antibody, *e.g.*, an intracellular antibody, which is specifically reactive with an epitope of the N-kinase polypeptide. In a preferred embodiment, the antibody is reactive with an epitope which includes the ATP binding domain of the N-kinase.

In another aspect, the invention features a fragment of the N-kinase polypeptide, for example, a fragment that includes at least 15, 20, 25, 30, 40, 50, 100, 150, or 200 contiguous amino acids of the N-kinase polypeptide. In a preferred embodiment, the fragment of the N-kinase polypeptide is able to elicit an immune response.

Pharmaceutical compositions, and packaged formulations, comprising a composition of the invention (*e.g.*, compound that modulates the activity of N-kinase) and a pharmaceutically acceptable carrier are also provided by the invention.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figures 1A-C are SDS-PAGE gels depicting the purification of the N kinase polypeptide. The N-kinase band at each stage of the purification is indicated by an asterisk. *Figure 1A* depicts a prominent 49 kDa band which binds strongly to a Cibacron Blue column and requires 1.5-1.75 M NaCl to be eluted. *Figure 1B* depicts the protein fractions obtained from the separation on a C4 hydrophobic-interaction column. Fractions 24-26 contain the N-kinase polypeptide. *Figure 1C* depicts the final stage of purification which was accomplished by SDS-PAGE. The band indicated by the asterisk coincided in its migration position with N-kinase activity, as visualized in a parallel gel assayed with the in-gel kinase method.

Figure 2 depicts the amino acid (SEQ ID NO:1) sequence of the human N-kinase. Direct matches between the purified protein and the published sequence are shown in blue. K65 (bold type) lies in the ATP-binding region of the kinase domain.

Detailed Description

The present invention provides methods and compositions for modulating the axonal outgrowth of central nervous system neurons, in particular mammalian central nervous system neurons. The invention is based, at least in part, on the isolation of a

highly purified form of the N-kinase polypeptide from a mammalian source and the discovery that this kinase plays an active role in the axonal outgrowth of CNS neurons, including mammalian CNS neurons, such as retinal ganglion neurons, or cortical pyramidal cells.

- 5 Accordingly, the present invention is directed to a method for treating a subject (e.g., a mammal, such as a human) suffering or prone to suffering from a condition characterized by aberrant axonal outgrowth of central nervous system neurons, (e.g., a condition characterized by a failure of injured central nervous system neurons to regrow their connections) by administering to the subject a compound that modulates the
10 activity of N-kinase, thereby treating the subject suffering or prone to suffering from a condition characterized by aberrant axonal outgrowth of central nervous system neurons.

- The methods of the invention for modulating, e.g., stimulating, the axonal outgrowth of central nervous system neurons can be used following damage or other injury to the CNS neurons (e.g., stroke, Traumatic Brain Injury, cerebral aneurism,
15 spinal cord injury and the like). The methods of the invention for modulating, e.g., inhibiting, the axonal outgrowth of CNS neurons can be used in neuroproliferative disorders where aberrant axonal outgrowth may occur, such as epilepsy (e.g., post-traumatic epilepsy) and neuropathic pain syndrome.

- As used herein, the term "N-kinase" includes all forms of N-kinase including but
20 not limited to human N-kinase, bovine N-kinase, murine N-kinase, rat N-kinase, and porcine N-kinase. The amino acid and nucleotide sequences of the human N-kinase are described in Zhou T-H. *et al.* (2000) *J. Biol. Chem.* 275(4):2513-2519 and in GenBank Accession Number AF083420, the contents of which are incorporated herein by reference. The amino acid sequence of the human N-kinase is shown in Figure 2 and in
25 SEQ ID NO:1. In a preferred embodiment, the term "N-kinase" includes the isoform of N-kinase that is inhibited by 6-thioguanine, that is activated by Mn^{+2} but not by Mg^{+2} or Ca^{+2} , and that has a molecular weight of approximately 49 kDa.

- As used herein, the language "a compound that modulates the activity of N-kinase" includes any compound which has the ability to modulate, e.g., stimulate or
30 inhibit, the activity of N-kinase as determined by, for example, the assays described herein. Such compounds are able to modulate one or more of the following: (a) the ability of N-kinase to phosphorylate a substrate, e.g., a histone HF-1 protein; (b) the ability of N-kinase to interact with, e.g., bind to, a non-N-kinase molecule, such as a downstream molecule in the axonal outgrowth signaling pathway; (c) the ability of N-
35 kinase to bind ATP or Mn^{+2} ; or (d) the ability of N-kinase to modulate the axonal outgrowth of central nervous system neurons.

 In a preferred embodiment, the compound that modulates the activity of N-kinase acts downstream of AF-1 or other growth factors in the axonal outgrowth signaling

pathway. The ability of the compound to act downstream of AF-1 or other growth factors in the axonal outgrowth signaling pathway may be determined using one of the assays described herein. For example, once a compound has been determined to be capable of stimulating the activity of N-kinase (e.g., stimulating the N-kinase dependent phosphorylation of a substrate), N-kinase may be contacted both with this compound and with 6-thioguanine. The inability of 6-thioguanine to inhibit the stimulatory effect of the compound would indicate that the compound is acting downstream of 6-thioguanine in the axonal outgrowth signaling pathway, whereas the ability of 6-thioguanine to inhibit the stimulatory effect of the compound would indicate that the compound is acting upstream (or at the same point) in the signaling pathway. Alternatively, once a compound has been determined to be capable of inhibiting the activity of N-kinase (e.g., inhibiting the N-kinase dependent phosphorylation of a substrate), N-kinase may be contacted both with this compound and with inosine. The inability of inosine to counteract the inhibitory effect of the compound would indicate that the compound is acting downstream of inosine in the axonal outgrowth signaling pathway, whereas the ability of inosine to counteract the inhibitory effect of the compound would indicate that the compound is acting upstream (or at the same point) in the signaling pathway.

In certain embodiments of the invention, the compound that modulates the activity of N-kinase can be any compound with the proviso that it is not a purine base (e.g., guanine, inosine, adenosine, and xanthine), such as a purine base linked to sugars, such as ribose, deoxyribose, and analogs and derivatives thereof. In certain other embodiments of the invention, the compound that modulates the activity of N-kinase can be any compound with the proviso that it is not a purine base analog or derivative thereof.

Examples of compounds that modulate the activity of N-kinase include small molecules, the N-kinase polypeptide or fragments thereof, an anti-N-kinase antibody, an antisense N-kinase nucleic acid molecule, a ribozyme, or the N-kinase gene or fragments thereof.

As used herein, the term "small molecule" includes, but is not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (*i.e.*, including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams

per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

As used herein, the language "modulating the axonal outgrowth of central nervous system neurons" is intended to include the capacity to stimulate or inhibit axonal outgrowth of central nervous system neurons to various levels, *e.g.*, to levels which allow for the treatment of a targeted CNS condition.

As used herein, the term "outgrowth" (*i.e.*, axonal outgrowth) refers to the process by which axons grow out of a CNS neuron. The outgrowth can result in a totally new axon or the repair of a partially damaged axon. Outgrowth is typically evidenced by extension of an axonal process of at least 5 cell diameters in length. Moreover, axonal outgrowth can be evidenced by GAP-43 expression (which can be detected by, for example, immunostaining).

As used herein, the term "CNS neurons" is intended to include the neurons of the brain and the spinal cord which are unresponsive to nerve growth factor (NGF). The term is not intended to include support or protection cells such as astrocytes, oligodendrocytes, microglia, ependyma and the like, nor is it intended to include peripheral nervous system (*e.g.*, somatic, autonomic, sympathetic or parasympathetic nervous system) neurons. Preferred CNS neurons are mammalian neurons, more preferably human neurons.

As used herein, the language "contacting" is intended to include both *in vivo* or *in vitro* methods of bringing a compound that modulates the activity of N-kinase into proximity with a CNS neuron, such that the compound that modulates the activity of N-kinase can modulate the outgrowth of axonal processes from the CNS neuron.

As used herein, the term "subject" is intended to include animals susceptible to conditions characterized by aberrant, *e.g.*, insufficient, axonal outgrowth of central nervous system neurons, preferably mammals, most preferably humans. In a preferred embodiment, the subject is a primate. In an even more preferred embodiment, the primate is a human. Other examples of subjects include dogs, cats, goats, and cows.

As used herein, the term "condition characterized by aberrant axonal outgrowth of central nervous system neurons" is intended to include a disease, disorder, or condition which is caused or characterized by an aberrant, *e.g.*, increased, insufficient, inadequate, or decreased, axonal outgrowth of central nervous system neurons. Such conditions directly or indirectly affect the normal functioning of the central nervous system (CNS). A condition characterized by aberrant, *e.g.*, insufficient, axonal outgrowth of central nervous system neurons includes, but is not limited to, an injury to the optic nerve, *e.g.*, affecting retinal ganglion cells; traumatic brain injury; stroke; cerebral aneurism; spinal cord injury, including monoplegia, diplegia, paraplegia, hemiplegia and quadriplegia; neuroproliferative disorders, *e.g.*, Alzheimer's disease,

dementias related to Alzheimer's disease (such as Pick's disease), Parkinson's and other Lewy diffuse body diseases, multiple sclerosis, amyotrophic lateral sclerosis, progressive supranuclear palsy, epilepsy, Jakob-Creutzfeldt disease, or AIDS related dementias; epilepsy, *e.g.*, posttraumatic brain injury; or neuropathic pain syndrome.

- 5 As used herein, the term "stroke" is art recognized and is intended to include sudden diminution or loss of consciousness, sensation, and voluntary motion caused by rupture or obstruction (*e.g.*, by a blood clot) of an artery of the brain.

- 10 As used herein, the term "Traumatic Brain Injury" is art recognized and is intended to include the condition in which, a traumatic blow to the head causes damage to the brain or connecting spinal cord, often without penetrating the skull. Usually, the initial trauma can result in expanding hematoma, subarachnoid hemorrhage, cerebral edema, raised intracranial pressure (ICP), and cerebral hypoxia, which can, in turn, lead to severe secondary events due to low cerebral blood flow (CBF).

- 15 In another aspect, the invention features a method for modulating, *e.g.*, stimulating or inhibiting, axonal outgrowth of a central nervous system neuron (such as a mammalian central nervous system neuron) by contacting the central nervous system neuron with a compound that modulates the activity of N-kinase, thereby modulating axonal outgrowth of the central nervous system neuron.

- 20 In yet another aspect, the invention features a method for modulating the axonal outgrowth of a central nervous system neuron in a subject, by administering to the subject a compound that modulates the activity of N-kinase, such that axonal outgrowth in the subject is modulated.

- 25 In yet another aspect, the invention features an isolated N-kinase polypeptide of the type that: (a) is present in neonatal brain tissue (*e.g.*, neonatal human, bovine, rat, or mouse brain tissue); (b) is inhibited by 6-thioguanine; (c) is activated by Mn^{+2} but not by Mg^{+2} or Ca^{+2} ; (d) has a molecular weight of approximately 49 kDa; and (e) is eluted from a Cibacron Blue column at a NaCl concentration of 1.5-1.75 M. As used herein, an "isolated" N-kinase polypeptide is substantially free (*i.e.*, greater than 95% free) of cellular material or other contaminating proteins from the cell or tissue source from which the N-kinase protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of N-kinase in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of N-kinase protein having less than about 20% (by dry weight) of non-N-kinase protein (*i.e.*, contaminating protein), more preferably less than about 10% of non-N-kinase protein, still more preferably less than about 5% of non-N-kinase protein, and most preferably less than about 3% non-N-kinase protein. When the N-
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In one embodiment, an assay is a cell-based assay in which a cell which expresses an N-kinase protein or biologically active portion thereof is contacted with a

test compound and the ability of the test compound to modulate N-kinase activity is determined. Determining the ability of the test compound to modulate N-kinase activity can be accomplished by monitoring, for example, the production of one or more specific metabolites in a cell which expresses N-kinase (see, *e.g.*, Saada *et al.* (2000) *Biochem Biophys. Res. Commun.* 269: 382-386). The cell, for example, can be of mammalian origin, *e.g.*, a neuronal cell.

Determining the ability of the test compound to modulate N-kinase activity can further be accomplished by, for example, determining the ability of N-kinase to phosphorylate a substrate. The ability of N-kinase to phosphorylate a substrate (*e.g.*, a histone HF-1 protein) can be determined by, for example, an *in vitro* kinase assay. Briefly, the N-kinase can be incubated with the substrate and radioactive ATP, *e.g.*, [γ - ^{32}P] ATP, in a buffer containing MnCl_2 , *e.g.*, 5 mM MnCl_2 . Following the incubation, the substrate can be immunoprecipitated or precipitated with TCA or collected on a filter (if no other kinases are present) and separated by SDS-polyacrylamide gel electrophoresis under reducing conditions, transferred to a membrane, *e.g.*, a PVDF membrane, and autoradiographed. The appearance of detectable bands on the autoradiograph indicates that the substrate has been phosphorylated. Alternatively, the in-gel assays described in Example 1 may be used to determine the ability of N-kinase to phosphorylate a substrate. Phosphoaminoacid analysis of the phosphorylated substrate can also be performed in order to determine which residues on the protein are phosphorylated. Briefly, the radiophosphorylated protein band can be excised from the SDS gel and subjected to partial acid hydrolysis. The products can then be separated by one-dimensional electrophoresis and analyzed on, for example, a phosphoimager and compared to ninhydrin-stained phosphoaminoacid standards.

The ability of the test compound to modulate N-kinase binding to a non-N-kinase molecule, such as a downstream molecule in the axonal outgrowth signaling pathway, can also be determined. Determining the ability of the test compound to modulate N-kinase binding to a non-N-kinase molecule can be accomplished, for example, by coupling the non-N-kinase molecule with a radioisotope or enzymatic label such that binding of the non-N-kinase molecule to N-kinase can be determined by detecting the labeled non-N-kinase molecule in a complex.

It is also within the scope of this invention to determine the ability of a test compound to interact with, *e.g.*, bind to, N-kinase or biologically active portions thereof. Preferred biologically active portions of the N-kinase proteins to be used in assays of the present invention include fragments which participate in interactions with non-N-kinase molecules, *e.g.*, fragments with high surface probability scores. Determining the ability of the test compound to bind N-kinase can be accomplished, for example, by coupling the compound with a radioisotope or enzymatic label such that binding of the compound

to N-kinase can be determined by detecting the labeled N-kinase compound in a complex. For example, test compounds can be labeled with ^{125}I , ^{35}S , ^{14}C , or ^3H , either directly or indirectly, and the radioisotope detected by direct counting of radioemission or by scintillation counting. Alternatively, test compounds can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

Determining the ability of a test compound to interact with N-kinase may also be accomplished without the labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of a compound with N-kinase without the labeling of either the compound or the N-kinase. McConnell, H. M. *et al.* (1992) *Science* 257:1906-1912. As used herein, a "microphysiometer" (*e.g.*, Cytosensor) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a compound and N-kinase.

Determining the ability of the test compound to bind to N-kinase or a biologically active portion thereof, can also be accomplished using a technology such as real-time Biomolecular Interaction Analysis (BIA). Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345 and Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705. As used herein, "BIA" is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore). Changes in the optical phenomenon of surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In an alternative embodiment, determining the ability of the test compound to modulate the activity of N-kinase can be accomplished by determining the ability of the N-kinase protein to further modulate the activity of a downstream effector of an N-kinase target molecule. For example, the activity of the effector molecule on an appropriate target can be determined or the binding of the effector to an appropriate target can be determined as previously described.

In more than one embodiment of the above assay methods of the present invention, it may be desirable to immobilize any of the reactants, *e.g.*, N-kinase or a non-N-kinase molecule, to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test compound to N-kinase, or interaction of N-kinase with a non-N-kinase molecule in the presence and absence of a test compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a

matrix. For example, glutathione-S-transferase/N-kinase fusion proteins or glutathione-S-transferase/target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed non-N-kinase molecule or N-kinase, and the mixture incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of N-kinase binding or activity determined using standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, either N-kinase or a non-N-kinase molecule can be immobilized utilizing conjugation of biotin and streptavidin.

Biotinylated N-kinase or non-N-kinase molecule can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with N-kinase or a non-N-kinase molecule but which do not interfere with the binding of N-kinase to its target non-N-kinase molecule can be derivatized to the wells of the plate, and unbound non-N-kinase molecule or N-kinase trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with N-kinase or a non-N-kinase molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with N-kinase or a non-N-kinase target molecule.

In another embodiment, modulators of N-kinase expression are identified in a method wherein a cell is contacted with a candidate compound and the expression of N-kinase mRNA or protein in the cell is determined. The level of expression of N-kinase mRNA or protein in the presence of the candidate compound is compared to the level of expression of N-kinase mRNA or protein in the absence of the candidate compound. The candidate compound can then be identified as a modulator of N-kinase expression based on this comparison. For example, when expression of N-kinase mRNA or protein is greater (statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of N-kinase mRNA or protein expression. Alternatively, when expression of N-kinase mRNA or protein is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of N-kinase

mRNA or protein expression. The level of N-kinase mRNA or protein expression in the cells can be determined by methods described herein for detecting N-kinase mRNA or protein.

In yet another aspect of the invention, the N-kinase proteins can be used as "bait proteins" in a two-hybrid assay or three-hybrid assay (see, e.g., U.S. Patent No. 5,283,317; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J. Biol. Chem.* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696; and Brent WO94/10300), to identify other proteins, which bind to or interact with N-kinase ("N-kinase-binding proteins" or "N-kinase-bp") and are involved in N-kinase activity. Such N-kinase-binding proteins are also likely to be involved in the propagation of signals by the N-kinase proteins or N-kinase targets as, for example, downstream elements of an N-kinase-mediated signaling pathway. Alternatively, such N-kinase-binding proteins are likely to be N-kinase inhibitors.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for an N-kinase protein is fused to a gene encoding the DNA binding domain of a known transcription factor (e.g., GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact, *in vivo*, forming an N-kinase-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g., LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with the N-kinase protein.

In another aspect, the invention pertains to a combination of two or more of the assays described herein. For example, a modulating compound can be identified using a cell-based or a cell free assay, and the ability of the compound to modulate the activity of an N-kinase protein can be confirmed *in vivo*, e.g., in an animal such as an animal model for a condition characterized by aberrant, e.g., insufficient axonal outgrowth of central nervous system neurons. Examples of such animal models are described in, for example, Benowitz *et al.* (1999) *PNAS* 96(23):13486-90. Epilepsy animal models are also known in the art.

This invention further pertains to novel compounds identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use a compound identified as described herein in an appropriate animal model.

For example, a compound identified as described herein (*e.g.*, an N-kinase modulating compound, an antisense N-kinase nucleic acid molecule, an N-kinase-specific antibody, or an N-kinase-binding partner) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such a compound. Alternatively, a compound identified as described herein can be used in an animal model to determine the mechanism of action of such a compound. Furthermore, this invention pertains to uses of novel compounds identified by the above-described screening assays for treatments as described herein.

In one embodiment, the N-kinase used in the methods of the invention is a human N-kinase, such as a recombinantly produced N-kinase. N-kinase, *e.g.*, human N-kinase, may be introduced into a recombinant expression vector using standard techniques and expressed in prokaryotic or eukaryotic cells. For example, N-kinase, *e.g.*, human N-kinase, can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, a recombinant expression vector containing N-kinase, *e.g.*, human N-kinase, can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

In another embodiment, the N-kinase used in the methods of the invention is a bovine N-kinase, such as an N-kinase which is purified from a bovine source, *e.g.*, neonatal bovine brain tissue, as described herein in, for instance, Example 1.

In another embodiment, the method of the invention further includes determining the ability of the test compound to modulate axonal outgrowth of a central nervous system neuron. Determining the ability of the test compound to modulate axonal outgrowth of a central nervous system neuron can be accomplished by, for example, using dissociated cultures of purified rat retinal ganglion cells. Dissociated cultures of purified rat retinal ganglion cells can, for example, be prepared by immunopanning as described in Barres *et al.*, *Neuron*, 1: 791-803, 1988, the contents of which are incorporated herein by reference. In brief, retinas from Sparague-Dawley rats can be dissociated using papain activated with cysteine. Macrophages are removed by incubation with an anti-rat macrophage antibody (Accurate) followed by immunopanning with an anti-rabbit IgG antibody. Ganglion cells are isolated by immunopanning with an anti-Thy-1 antibody, then dislodged with trypsin for use in low-density cultures. Rat retinal ganglion cells are maintained at 37° C in a CO₂ incubator using the same medium described above except for the presence of 30 mM bicarbonate.

Samples are plated in quadruplicate in randomized positions of a 24-well plate, contacted with the test compound, and the code is concealed to ensure that growth is

evaluated in a blinded fashion. Each experiment may contain 4 wells of a negative control (media plus supplements only) and 4 wells of a positive control (*e.g.*, a standardized AF-1 sample of known activity). Growth and survival are assessed after 6 days for all ganglion cells in 25 consecutive fields of each well using phase contrast microscopy at 400X magnification (c. 150 ganglion cells counted per well). Extension of a process 5 cell diameters in length is used as the criterion for growth, since it clearly distinguishes stimulated cells from negative controls (Schwalb *et al.*, 1995). After the completion of counting, the code is broken, the data tabulated, and means and standard errors are calculated for the 4 replicate wells of each sample using Cricket Graph (CA Associates, Islandia, NY). Data are normalized by subtracting the growth in the negative controls (usually 4-5%) and dividing by the net growth in the positive controls. Goldfish retinal ganglion cells (Benowitz *et al.* (1998) *J. Biol. Chem.* 273(45):29626-34) as well as mixtures of rat and goldfish ganglion cells may also be used.

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Compounds That Modulate Axonal Outgrowth Of A Central Nervous System Neuron

In another aspect, the invention features a compound that modulates axonal outgrowth of a central nervous system neuron identified by any of the foregoing methods.

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In one embodiment, the compound that modulates axonal outgrowth of a central nervous system neuron is an antisense N-kinase nucleic acid molecule. An "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to the entire N-kinase coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding an N-kinase. The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues. In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding N-kinase. The term "noncoding region" refers to 5' and 3' sequences which flank the coding region that are not translated into amino acids (*i.e.*, also referred to as 5' and 3' untranslated regions).

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Given the coding strand sequence encoding N-kinase, antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of N-kinase mRNA, but more preferably is an oligonucleotide which is antisense to only a

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portion of the coding or noncoding region of N-kinase mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of N-kinase mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding an N-kinase protein to thereby inhibit expression of the protein, *e.g.*, by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An example of a route of administration of antisense nucleic acid molecules of the invention include direct injection at a tissue site, *e.g.*, in the brain. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that

they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*, by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense N-kinase nucleic acid molecule may be an α -anomeric nucleic acid molecule. An α -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gaultier *et al.* (1987) *Nucleic Acids Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-*o*-methylribonucleotide (Inoue *et al.* (1987) *Nucleic Acids Res.* 15:6131-6148) or a chimeric RNA-DNA analogue (Inoue *et al.* (1987) *FEBS Lett.* 215:327-330).

In still another embodiment, the compound that modulates axonal outgrowth of a central nervous system neuron is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes (described in Haselhoff and Gerlach (1988) *Nature* 334:585-591)) can be used to catalytically cleave N-kinase mRNA transcripts to thereby inhibit translation of N-kinase mRNA. A ribozyme having specificity for an N-kinase-encoding nucleic acid can be designed based upon the nucleotide sequence of an N-kinase cDNA. For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in an N-kinase-encoding mRNA. See, *e.g.*, Cech *et al.* U.S. Patent No. 4,987,071; and Cech *et al.* U.S. Patent No. 5,116,742. Alternatively, N-kinase mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, *e.g.*, Bartel, D. and Szostak, J.W. (1993) *Science* 261:1411-1418.

Alternatively, N-kinase gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the N-kinase (*e.g.*, the N-kinase promoter and/or enhancers) to form triple helical structures that prevent transcription of the N-kinase gene in target cells. See generally, Helene, C. (1991) *Anticancer Drug Des.* 6(6): 569-84; Helene, C. *et al.* (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L.J. (1992) *Bioassays* 14(12):807-15.

In still another embodiment, the compound that modulates axonal outgrowth of a central nervous system neuron is an anti-N-kinase antibody. A full-length N-kinase protein or, alternatively, antigenic peptide fragments of N-kinase may be used as

immunogens to generate anti-N-kinase antibodies. The antigenic peptide of N-kinase comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO:1 and encompasses an epitope of N-kinase such that an antibody raised against the peptide forms a specific immune complex with the N-kinase protein. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues.

Preferred epitopes encompassed by the antigenic peptide are regions of N-kinase that are located on the surface of the protein, *e.g.*, hydrophilic regions, as well as regions with high antigenicity.

An N-kinase immunogen typically is used to prepare antibodies by immunizing a suitable subject, (*e.g.*, rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed N-kinase protein or a chemically synthesized N-kinase polypeptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic N-kinase preparation induces a polyclonal anti-N-kinase antibody response.

The term "antibody" as used herein includes immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, such as an N-kinase. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and F(ab')₂ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind N-kinase molecules. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of N-kinase. A monoclonal antibody composition thus typically displays a single binding affinity for a particular N-kinase protein with which it immunoreacts.

Polyclonal anti-N-kinase antibodies can be prepared as described above by immunizing a suitable subject with an N-kinase immunogen. The anti-N-kinase antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized N-kinase. If desired, the antibody molecules directed against N-kinase can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, *e.g.*, when the anti-N-kinase antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare

monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497) (see also, Brown *et al.* (1981) *J. Immunol.* 127:539-46; Brown *et al.* (1980) *J. Biol. Chem.* 255:4980-83; Yeh *et al.* (1976) *Proc. Natl. Acad. Sci. USA* 76:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal antibody hybridomas is well known (see generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension* 10 *In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; M. L. Gefter *et al.* (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal immunized with an N-kinase immunogen as described above, and the culture supernatants of the resulting hybridoma 15 cells are screened to identify a hybridoma producing a monoclonal antibody that binds N-kinase.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-N-kinase monoclonal antibody (see, *e.g.*, G. Galfre *et al.* (1977) *Nature* 266:55052; Gefter *et al.* 20 *Somatic Cell Genet.*, cited *supra*; Lerner, *Yale J. Biol. Med.*, cited *supra*; Kenneth, *Monoclonal Antibodies*, cited *supra*). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the lymphocytes. For example, murine hybridomas can be made 25 by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a fusion partner according to standard techniques, *e.g.*, 30 the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma cells (unfused splenocytes die after several days because 35 they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind N-kinase, *e.g.*, using a standard ELISA assay.

Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

In one embodiment, the compound that modulates axonal outgrowth of a central nervous system neuron is an intracellular antibody specific for N-kinase. The use of intracellular antibodies to inhibit protein function in a cell is known in the art (see *e.g.*, Carlson, J. R. (1988) *Mol. Cell. Biol.* 8:2638-2646; Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Werge, T.M. *et al.* (1990) *FEBS Letters* 274:193-198; Carlson, J.R. (1993) *Proc. Natl. Acad. Sci. USA* 90:7427-7428; Marasco, W.A. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:7889-7893; Biocca, S. *et al.* (1994) *Bio/Technology* 12:396-399; Chen, S-Y. *et al.* (1994) *Human Gene Therapy* 5:595-601; Duan, L *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5075-5079; Chen, S-Y. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5932-5936; Beerli, R.R. *et al.* (1994) *J. Biol. Chem.* 269:23931-23936; Beerli, R.R. *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:666-672; Mhashilkar, A.M. *et al.* (1995) *EMBO J.* 14:1542-1551; Richardson, J.H. *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:3137-3141; PCT Publication No. WO 94/02610 by Marasco *et al.*; and PCT Publication No. WO 95/03832 by Duan *et al.*).

To inhibit protein activity using an intracellular antibody, a recombinant expression vector is prepared which encodes the antibody chains in a form such that, upon introduction of the vector into a cell, the antibody chains are expressed as a functional antibody in an intracellular compartment of the cell.

In still another embodiment, the compound that modulates axonal outgrowth of a central nervous system neuron is a small molecule. As used herein, the term "small molecule" includes, but is not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (*i.e.*, including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

In still another embodiment, the compound that modulates axonal outgrowth of a central nervous system neuron is an N-kinase polypeptide or portion thereof, *e.g.*, a fragment that includes at least 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 150, or 200 contiguous amino acids of the N-kinase polypeptide. For example, the compound could comprise the constitutively active catalytic domain of the N-kinase.

In still another embodiment, the compound that modulates axonal outgrowth of a central nervous system neuron is an N-kinase gene or portion thereof.

Pharmaceutically Acceptable Formulations

5 Pharmaceutical compositions, and packaged formulations, comprising a composition of the invention (*e.g.*, compound that modulates the activity of N-kinase) and a pharmaceutically acceptable carrier are also provided by the invention. In the method of the invention, the compound that modulates the activity of N-kinase can be administered in a pharmaceutically acceptable formulation. Such pharmaceutically acceptable formulation typically includes the compound that
10 modulates the activity of N-kinase as well as a pharmaceutically acceptable carrier(s) and/or excipient(s). As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and anti fungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. For example, the carrier can be suitable for injection into the cerebrospinal fluid. Excipients
15 include pharmaceutically acceptable stabilizers and disintegrants. The present invention pertains to any pharmaceutically acceptable formulations, including synthetic or natural polymers in the form of macromolecular complexes, nanocapsules, microspheres, or beads, and lipid-based formulations including oil-in-water emulsions, micelles, mixed micelles, synthetic membrane vesicles, and resealed erythrocytes.

20 In one embodiment, the pharmaceutically acceptable formulations comprise a polymeric matrix. The terms "polymer" or "polymeric" are art-recognized and include a structural framework comprised of repeating monomer units which is capable of delivering a compound that modulates the activity of N-kinase such that treatment of a targeted condition, *e.g.*, a CNS injury, occurs. The terms also include co-polymers and
25 homopolymers *e.g.*, synthetic or naturally occurring. Linear polymers, branched polymers, and cross-linked polymers are also meant to be included.

For example, polymeric materials suitable for forming the pharmaceutically acceptable formulation employed in the present invention, include naturally derived polymers such as albumin, alginate, cellulose derivatives, collagen, fibrin, gelatin, and
30 polysaccharides, as well as synthetic polymers such as polyesters (PLA, PLGA), polyethylene glycol, poloxomers, polyanhydrides, and pluronics. These polymers are biocompatible with the nervous system, including the central nervous system, they are biodegradable within the central nervous system without producing any toxic byproducts of degradation, and they possess the ability to modify the manner and duration of
35 compound that modulates the activity of N-kinase release by manipulating the polymer's kinetic characteristics. As used herein, the term "biodegradable" means that the polymer will degrade over time by the action of enzymes, by hydrolytic action and/or by other similar mechanisms in the body of the subject. As used herein, the term "biocompatible"

means that the polymer is compatible with a living tissue or a living organism by not being toxic or injurious and by not causing an immunological rejection.

Polymers can be prepared using methods known in the art (Sandler, S. R.; Karo, W. *Polymer Syntheses*; Harcourt Brace: Boston, 1994; Shalaby, W.; Ikada, Y.; Langer, R.; Williams, J. *Polymers of Biological and Biomedical Significance (ACS Symposium Series 540)*; American Chemical Society: Washington, DC, 1994). Polymers can be designed to be flexible; the distance between the bioactive side-chains and the length of a linker between the polymer backbone and the group can be controlled. Other suitable polymers and methods for their preparation are described in U.S. Patent Nos. 5,455,044 and 5,576,018, the contents of which are incorporated herein by reference.

The polymeric formulations can be formed by dispersion of the compound that modulates the activity of N-kinase within liquefied polymer, as described in U.S. Pat. No. 4,883,666, the teachings of which are incorporated herein by reference or by such methods as bulk polymerization, interfacial polymerization, solution polymerization and ring polymerization as described in Odian G., *Principles of Polymerization and ring opening polymerization*, 2nd ed., John Wiley & Sons, New York, 1981, the contents of which are incorporated herein by reference. The properties and characteristics of the formulations are controlled by varying such parameters as the reaction temperature, concentrations of polymer and compound that modulates the activity of N-kinase, types of solvent used, and reaction times.

The compound that modulates the activity of N-kinase can be encapsulated in one or more pharmaceutically acceptable polymers, to form a microcapsule, microsphere, or microparticle, terms used herein interchangeably. Microcapsules, microspheres, and microparticles are conventionally free-flowing powders consisting of spherical particles of 2 millimeters or less in diameter, usually 500 microns or less in diameter. Particles less than 1 micron are conventionally referred to as nanocapsules, nanoparticles or nanospheres. For the most part, the difference between a microcapsule and a nanocapsule, a microsphere and a nanosphere, or microparticle and nanoparticle is size; generally there is little, if any, difference between the internal structure of the two. In one aspect of the present invention, the mean average diameter is less than about 45 μ m, preferably less than 20 μ m, and more preferably between about 0.1 and 10 μ m.

In another embodiment, the pharmaceutically acceptable formulations comprise lipid-based formulations. Any of the known lipid-based drug delivery systems can be used in the practice of the invention. For instance, multivesicular liposomes (MVL), multilamellar liposomes (also known as multilamellar vesicles or "MLV"), unilamellar liposomes, including small unilamellar liposomes (also known as unilamellar vesicles or "SUV") and large unilamellar liposomes (also known as large unilamellar vesicles or "LUV"), can all be used so long as a sustained release rate of the encapsulated

compound that modulates the activity of N-kinase or analogue thereof can be established. In one embodiment, the lipid-based formulation can be a multivesicular liposome system. Methods of making controlled release multivesicular liposome drug delivery systems is described in PCT Application Serial Nos. US96/11642, US94/12957
 5 and US94/04490, the contents of which are incorporated herein by reference.

The composition of the synthetic membrane vesicle is usually a combination of phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used.

Examples of lipids useful in synthetic membrane vesicle production include
 10 phosphatidylglycerols, phosphatidylcholines, phosphatidylserines, phosphatidylethanolamines, sphingolipids, cerebrosides, and gangliosides. Preferably phospholipids including egg phosphatidylcholine, dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylglycerol, and dioleoylphosphatidylglycerol are used.

15 In preparing lipid-based vesicles containing a compound that modulates the activity of N-kinase or analogue thereof, such variables as the efficiency of compound that modulates the activity of N-kinase encapsulation, lability of the compound that modulates the activity of N-kinase, homogeneity and size of the resulting population of vesicles, compound that modulates the activity of N-kinase-to-lipid ratio, permeability,
 20 instability of the preparation, and pharmaceutical acceptability of the formulation should be considered (see Szoka, *et al.*, *Annual Reviews of Biophysics and Bioengineering*, 9:467, 1980; Deamer, *et al.*, in *Liposomes*, Marcel Dekker, New York, 1983, 27; and Hope, *et al.*, *Chem. Phys. Lipids*, 40:89, 1986, the contents of which are incorporated herein by reference).

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Administration of the Pharmaceutically Acceptable Formulation

The pharmaceutically acceptable formulations of the invention are administered such that the compound that modulates the activity of N-kinase, or analogue thereof, comes into contact with central nervous system neurons to thereby modulate the axonal
 30 outgrowth thereof. Both local and systemic administration of the formulations are contemplated by the invention, although local administration may be preferable to achieve effective local concentrations of the compound that modulates the activity of N-kinase, or analogue, as well as to avoid possible side effects from systemic administration of the agent. In one embodiment, the compound that modulates the
 35 activity of N-kinase is administered by introduction into the central nervous system of the subject, *e.g.*, into the cerebrospinal fluid of the subject. In certain aspects of the invention, the compound that modulates the activity of N-kinase is introduced intrathecally, *e.g.*, into a cerebral ventricle, the lumbar area, or the cisterna magna. In

another aspect, the compound that modulates the activity of N-kinase is introduced intraocularly, to thereby contact retinal ganglion cells.

The pharmaceutically acceptable formulations can easily be suspended in aqueous vehicles and introduced through conventional hypodermic needles or using infusion pumps. Prior to introduction, the formulations can be sterilized with, preferably, gamma radiation or electron beam sterilization, described in US Patent No. 436,742 the contents of which are incorporated herein by reference.

In one embodiment, the compound that modulates the activity of N-kinase formulation described herein is administered to the subject in the period from the time of, for example, an injury to the CNS up to about 100 or 200 hours after the injury has occurred, for example, within 48, 36, 24, 12, or 6 hours from the time of injury. In another embodiment, the compound that modulates the activity of N-kinase is administered to a subject suffering from a chronic injury to the CNS. Accordingly, the compound is administered to the subject over the subject's life time.

In another embodiment of the invention, the compound that modulates the activity of N-kinase formulation is administered into a subject intrathecally. As used herein, the term "intrathecal administration" is intended to include delivering a compound that modulates the activity of N-kinase formulation directly into the cerebrospinal fluid of a subject, by techniques including lateral cerebroventricular injection through a burrhole or cisternal or lumbar puncture or the like (described in Lazorthes *et al.* Advances in Drug Delivery Systems and Applications in Neurosurgery, 143-192 and Omayya *et al.*, Cancer Drug Delivery, 1: 169-179, the contents of which are incorporated herein by reference). The term "lumbar region" is intended to include the area between the third and fourth lumbar (lower back) vertebrae. The term "cisterna magna" is intended to include the area where the skull ends and the spinal cord begins at the back of the head. The term "cerebral ventricle" is intended to include the cavities in the brain that are continuous with the central canal of the spinal cord. Administration of a compound that modulates the activity of N-kinase to any of the above mentioned sites can be achieved by direct injection of the compound that modulates the activity of N-kinase formulation or by the use of infusion pumps. For injection, the compound that modulates the activity of N-kinase formulation of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the compound that modulates the activity of N-kinase formulation may be formulated in solid form and re-dissolved or suspended immediately prior to use. Lyophilized forms are also included. The injection can be, for example, in the form of a bolus injection or continuous infusion (*e.g.*, using infusion pumps) of the compound that modulates the activity of N-kinase formulation.

In one embodiment of the invention, the compound that modulates the activity of N-kinase formulation is administered by lateral cerebro ventricular injection into the brain of a subject, preferably within 100 hours of when an injury (resulting in a condition characterized by aberrant axonal outgrowth of central nervous system neurons) occurs (*e.g.*, within 6, 12, or 24 hours of the time of the injury). The injection can be made, for example, through a burr hole made in the subject's skull. In another embodiment, the formulation is administered through a surgically inserted shunt into the cerebral ventricle of a subject, preferably within 100 hours of when an injury occurs (*e.g.*, within 6, 12 or 24 hours of the time of the injury). For example, the injection can be made into the lateral ventricles, which are larger, even though injection into the third and fourth smaller ventricles can also be made. In yet another embodiment, the compound that modulates the activity of N-kinase formulation is administered by injection into the cisterna magna, or lumbar area of a subject, preferably within 100 hours of when an injury occurs (*e.g.*, within 6, 12, or 24 hours of the time of the injury).

In another embodiment of the invention, the compound that modulates the activity of N-kinase formulation is administered to a subject at the site of injury, preferably within 100 hours of when an injury occurs (*e.g.*, within 6, 12, or 24 hours of the time of the injury).

Duration and Levels of administration

In a preferred embodiment of the method of the invention, the compound that modulates the activity of N-kinase, or analog thereof, is contacted with CNS neurons for an extended period of time to effect modulation of axonal outgrowth. Sustained contact with the compound that modulates the activity of N-kinase, or analog, can be achieved by, for example, repeated administration of the compound that modulates the activity of N-kinase or analog over a period of time, such as one week, several weeks, one month or longer. More preferably, the pharmaceutically acceptable formulation used to administer the compound that modulates the activity of N-kinase, or analog, provides sustained delivery, *e.g.*, "slow release" of the compound that modulates the activity of N-kinase, or analog, to a subject. For example, the formulation may deliver the compound that modulates the activity of N-kinase, or analog, for at least one, two, three, or four weeks after the pharmaceutically acceptable formulation is administered to the subject. Preferably, a subject to be treated in accordance with the present invention is treated with the compound that modulates the activity of N-kinase, or analog, for at least 30 days (either by repeated administration or by use of a sustained delivery system, or both).

As used herein, the term "sustained delivery" is intended to include continual delivery of a compound that modulates the activity of N-kinase or analogue thereof *in*

vivo over a period of time following administration, preferably at least several days, a week, several weeks, one month or longer. Sustained delivery of the compound that modulates the activity of N-kinase or analogue thereof can be demonstrated by, for example, the continued therapeutic effect of the compound that modulates the activity of N-kinase or analogue thereof over time (*e.g.*, sustained delivery of the compound that modulates the activity of N-kinase or analogue thereof can be demonstrated by continued outgrowth or by continued inhibition of outgrowth of CNS neurons over time). Alternatively, sustained delivery of the compound that modulates the activity of N-kinase or analogue thereof may be demonstrated by detecting the presence of the compound that modulates the activity of N-kinase or analogue thereof *in vivo* over time.

Preferred approaches for sustained delivery include use of a polymeric capsule or a minipump to deliver the formulation. Polymeric capsules can be prepared as described herein. Implantable infusion pump systems (*e.g.*, Infusaid; see *e.g.*, Zierski, J. *et al.* (1988) *Acta Neurochem. Suppl.* 43:94-99; Kanoff, R.B. (1994) *J. Am. Osteopath. Assoc.* 94:487-493) and osmotic pumps (sold by Alza Corporation) are available in the art. Another mode of administration is via an implantable, externally programmable infusion pump. Suitable infusion pump systems and reservoir systems are also described in U.S. Patent No. 5, 368,562 by Blomquist and U.S. Patent No. 4,731,058 by Doan, developed by Pharmacia Deltec Inc.

The pharmaceutical formulation, used in the method of the invention, contains a therapeutically effective amount of the compound that modulates the activity of N-kinase or analogue thereof. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired result. A therapeutically effective amount of the compound that modulates the activity of N-kinase or analogue thereof may vary according to factors such as the disease state, age, and weight of the subject, and the ability of the compound that modulates the activity of N-kinase or analogue thereof (alone or in combination with one or more other agents) to elicit a desired response in the subject. Dosage regimens may be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental effects of the compound that modulates the activity of N-kinase or analogue thereof are outweighed by the therapeutically beneficial effects. A non-limiting dosage range is about 5 μM - 1000 μM , although the particular optimal dosage will vary depending upon, among other factors, the particular compound that modulates the activity of N-kinase, or analogue thereof, used.

It is to be noted that dosage values may vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of

the compound that modulates the activity of N-kinase or analogue thereof and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed invention.

The invention, in another embodiment, provides a pharmaceutical composition
5 consisting essentially of a compound that modulates the activity of N-kinase and a pharmaceutically acceptable carrier, as well as methods of use thereof to modulate axonal outgrowth by contacting CNS neurons with the composition. By the term "consisting essentially of" is meant that the pharmaceutical composition does not contain
10 any other modulators of neuronal growth such as, for example, nerve growth factor (NGF). In one embodiment, the pharmaceutical composition of the invention can be provided as a packaged formulation. The packaged formulation may include a pharmaceutical composition of the invention in a container and printed instructions for administration of the composition for treating a subject having a disorder associated with
15 an injury of central nervous system neurons, *e.g.*, an injury to retinal ganglion cells, a spinal cord injury or a traumatic brain injury. Use of the compound that modulates the activity of N-kinases, and analogues thereof, of the invention in the manufacture of a medicament for modulating the outgrowth of CNS neurons (*e.g.*, mammalian CNS neurons) is also encompassed by the invention.

20 *In vitro* Treatment of CNS Neurons

CNS neurons can further be contacted with a compound that modulates the activity of N-kinase, *in vitro* to modulate axonal outgrowth *in vitro*. Accordingly, CNS neuron cells can be isolated from a subject and grown *in vitro*, using techniques well known in the art, and then treated in accordance with the present invention to modulate
25 axonal outgrowth. Briefly, a CNS neuron cell culture can be obtained by allowing neuron cells to migrate out of fragments of neural tissue adhering to a suitable substrate (*e.g.*, a culture dish) or by disaggregating the tissue, *e.g.*, mechanically or enzymatically, to produce a suspension of CNS neuron cells. For example, the enzymes trypsin, collagenase, elastase, hyaluronidase, DNase, pronase, dispase, or various combinations
30 thereof can be used. Trypsin and pronase give the most complete disaggregation but may damage the cells. Collagenase and dispase give a less complete disaggregation but are less harmful. Methods for isolating tissue (*e.g.*, neural tissue) and the disaggregation of tissue to obtain cells (*e.g.*, CNS neuron cells) are described in Freshney R. I., Culture of Animal Cells, A Manual of Basic Technique, Third Edition, 1994, the contents of
35 which are incorporated herein by reference.

Such cells can be subsequently contacted with a compound that modulates the activity of N-kinase in amounts and for a duration of time as described above. Once modulation of axonal outgrowth has been achieved in the CNS neuron cells, these cells

can be re-administered to the subject, *e.g.*, by implantation. It is preferred that the cells are not allowed to differentiate extensively *in vitro*, as cells that integrate most successfully in a subject are primitive cells.

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The invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and the
10 Sequence Listing are hereby incorporated by reference.

Examples

Example I. Isolation and Characterization of the N-kinase Polypeptide

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Neocortical gray matter from bovine brain was homogenized in buffer containing protease and phosphatase inhibitors, and particulate material was centrifuged down. The soluble fraction from approximately 1 kg of tissue was used as the starting material for isolation of the kinase.

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During the purification process, kinase activity was monitored using an in-gel kinase method. In this method, a histone HF-1 substrate protein is polymerized into a 10% polyacrylamide gel before the samples to be analyzed are electrophoresed in the gel. Following completion of the electrophoresis, proteins are partially renatured with guanidium isothiocyanate and incubated in the presence [³²P]-ATP plus Mn²⁺, with or
25 without 6-TG present. (Activation by Mn²⁺, but not by Mg²⁺ or Ca²⁺, is a distinctive property of N-kinase). The purification of the kinase was monitored by looking for a 6-TG-inhibitable radioactive band corresponding to the site where the kinase phosphorylates the HF-1 substrate in the gel.

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In the first step of the purification process the starting material was subjected to cation-exchange chromatography (using a Fast-S column, Pharmacia). A 6-TG inhibitable kinase activity bound strongly to this column and eluted with 0.3 M NaCl. The cation-exchange column fraction containing N-kinase was subsequently separated on a Cibacron Blue column (Pharmacia), which allows for the separation of adenine nucleotide-binding proteins. A 6-TG inhibitable, 47-50 kDa polypeptide bound strongly
35 and required a NaCl concentration of 1.5 M NaCl for elution (see Figure 1A).

The eluted fraction containing the N-kinase polypeptide was, then, subjected to reversed-phase chromatography with a C4 hydrophobic interaction column (Pharmacia). Briefly, the Cibacron Blue column fraction containing the N-kinase was applied to the

C4 column and eluted with a gradient of increasing acetonitrile-isopropanol concentration. Evaluation of the kinase activity of the column fractions by in-gel kinase assays showed that the 6-TG-inhibitable, HF-1-phosphorylating activity eluted in fractions 24-26 (Figure 1B). To achieve a higher level of purification, these fractions were pooled, re-applied to the same column, and the separation was repeated with a gradient of increasing acetonitrile-isopropanol concentration. The N-kinase polypeptide, again, eluted at fractions 24-26.

The column fractions containing the highest concentrations of N-kinase were lyophilized and applied to a 10% polyacrylamide SDS gel. A small portion of the sample was run on a parallel gel to carry out in-gel kinase assays. A band at 49 kDa was clearly visible after staining the gel with Coomassie blue; this coincided in its migration position with the 6-TG-inhibitable kinase activity. This band was cut out and it was verified that it contained HF-1-phosphorylating activity.

The gel band containing the N-kinase polypeptide was then subjected to partial proteolytic digestion, the proteolytic fragments were analyzed by mass spectroscopy and the masses of the fragments compared to those of various known peptides using the process described in, for example, Eng J.K. *et al.* (1994) *J. Am. Soc. Mass. Spectrom.* 5:976-989; Chittum H.S. *et al.* (1998) *Biochemistry* 37:10866-870; and LeRoy G. *et al.* (1998) *Science* 282:1900-04, the contents of which are incorporated herein by reference.

This analysis revealed that N-kinase is an isoform of MST-3, *i.e.*, either MST-3 itself, MST-3b, or an as yet undefined isoform. These proteins are members of the STE family of serine-threonine kinases that are found throughout the animal kingdom. STE family members are generally components of modular signaling cassettes that are involved in various aspects of cellular differentiation. Figure 2 depicts the amino acid sequence of the N-kinase. Direct matches between the purified protein and the published sequence are shown in blue. K65 (bold type) lies in the ATP-binding region of the kinase domain.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following

5 claims.

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claims.